tor (TNF)[22,38-41]. This factor causes necrosis of MethA(a) coma *in vivo*; it is also active in causing lysis of murine L-929 ls *in vitro*[22,39]. TNF activity on L-929 cells is not inhibited neutralizing antibodies specific for lymphotoxin[13], however, d appears to have distinct biochemical properties[39,40]. Furthermore, the lymphotoxin cDNA probe failed to hybridize on rthern blots to mRNA from induced macrophages producing olytic activity (Fig. 2). The isolation of a TNF DNA clone ther demonstrates that lymphotoxin and TNF are distinct lecules (see accompanying article[42]).

A cytolytic factor derived from B-cell lines which displays *in ro* and *in vivo* anticellular activity has been described[43]; this s been designated 'tumour necrosis factor' based on its activity the MethA sarcoma assay. This activity probably results from mphotoxin, however, because it has similar biological ivities, biochemical properties and is made by the cell line PMI-1788) used in this study for the purification of lymotoxin. Natural killer cells also can be induced to secrete an ticellular factor[44,45]. The lymphotoxin gene probe and lymotoxin-specific antibodies will be useful in determining the relationship of this natural killer cytotoxic factor to lymphotoxin.

Lymphotoxin has been reported to act synergistically with α-interferon[46] and γ-interferon[13,14,47] *in vitro* and *in vivo*. The potent antitumour activity of γ-interferon or lymphotoxin in natural preparations may be a result of the synergistic activity when both lymphokines are present[13]. The ready availability of lymphotoxin produced via recombinant methods will aid the biological characterization of this anticellular lymphokine. It will also help to define the antitumour mechanism of lymphotoxin, as well as its role *in vivo* in the regulation of the immune system and its interaction with other lymphokines.

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Human tumour necrosis factor: precursor tructure, expression and homology to lymphotoxin

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uman tumour necrosis factor has about 30% homology in its amino acid sequence with lymphotoxin, a lymphokine that s similar biological properties. Recombinant tumour necrosis factor can be obtained by expression of its complementary NA in Escherichia coli *and induces the haemorrhagic necrosis of transplanted methylcholanthrene-induced sarcomas syngeneic mice.*

MOUR necrosis factor (TNF) has been associated with *in ro* and *in vivo* killing of tumour cells. This activity was covered originally in the sera of mice and rabbits injected t with *Mycobacterium bovis* strain bacillus Calmette-Guérin CG) or other immunostimulatory agents, and subsequently with endotoxin[1,2]. Serum from such animals causes haemorrhagic necrosis and in some cases complete regression of certain transplanted tumours in mice[1,2]. TNF-like activity has also been detected in the media of BCG/endotoxin-induced monocyte cultures (reviewed in ref. 2) and mitogen-stimulated peripheral

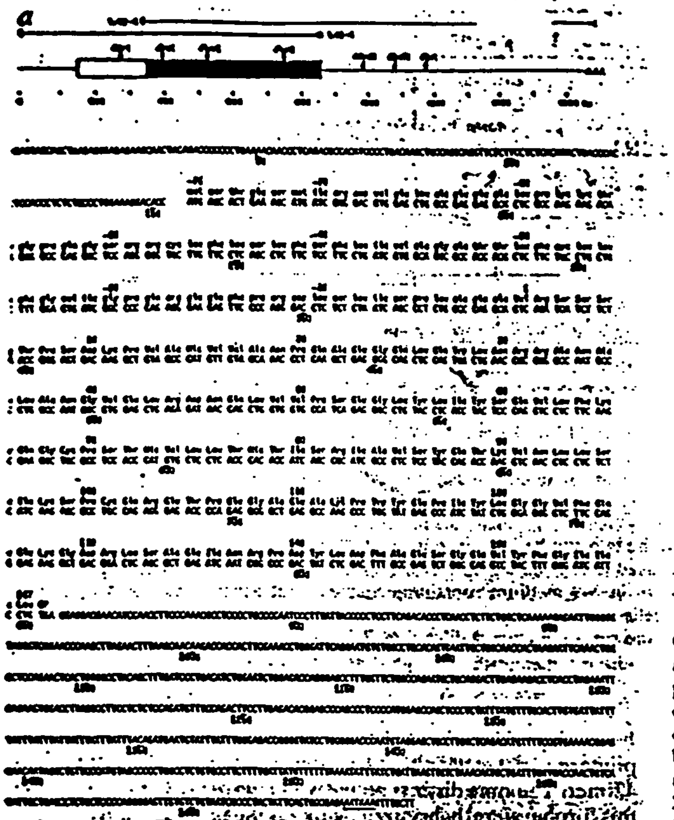

Fig. 1 TNF cDNA sequences and predicted amino acid sequence. *a*, Schematic representation of human TNF cDNA clones. Overlapping clones λ42-4 and λ16-4 used in sequence determination and a schematic diagram of the complete cDNA structure are shown. Line, untranslated sequences; boxes, coding sequences; white portion, sequences encoding the signal peptide; shaded regions code for mature TNF. The black box on the 3' end of clone λ16-4 indicates that this clone was obtained by specific priming. *b*, Nucleotide sequence and deduced amino acid sequence of human TNF cDNA. Numbers above each line refer to amino acid positions and numbers below each line refer to nucleotide positions. The amino acid labelled 1 represents the first amino acid of mature TNF[14]. The 76 amino acids preceding this position are indicated by lower case lettering. Sequence underlined indicates the polyadenylation recognition site[37].

Methods: *a*, Total RNA was extracted[38] from HL-60 cultures 4 h after PMA induction and poly(A)-containing RNA was purified on oligo(dT)-cellulose[43]. Double-stranded cDNA was prepared by oligo(dT) priming[19] using 7.5 μg mRNA and fractionated on a 6% polyacrylamide gel. 700 ng cDNA >600 bp was recovered by electroelution. Synthetic *Eco*RI adaptors[44] were ligated to 20 ng cDNA before ligating into λgt10 (ref. 26). 200,000 cDNA clones were obtained. The same conditions were used to prepare a specifically-primed cDNA library of 200,000 clones using as primer the hexadecanucleotide dTGGATGTTCGTCCTCC (complementary to nucleotides 855–870). Plaque screening[45], ^{32}P-radiolabelling of synthetic 42-mer probe[25] and hybridizations[22] were performed. *b*, DNA sequencing was performed by the dideoxynucleotide chain termination procedure[46-48]. The cDNA insert of λ42-4 consists of nucleotides 337–1643 and the cDNA insert of λ16-4 consists of nucleotides 1–870.

blood leukocytes (PBLs)[3].

TNF activity is cytolytic or cytostatic against many transformed cell lines *in vitro* without obvious species specificity, yet has no known effect on normal mouse embryo fibroblasts or non-transformed cell lines[1,2,4-8]. Activated macrophages may constitute the major cellular origin of TNF[1,3,9,10], providing an important criterion for distinguishing this factor from the lymphoid cell-derived cytotoxin, lymphotoxin[11]. The primary structure of lymphotoxin was determined recently by protein sequencing[12] and complementary DNA cloning (see accompanying article[13]).

Table 1 Human TNF production by various cell populations and cell lines

Cell source	Inducing agent(s)	Cytotoxic activity (U ml^{-1}) TNF	Lymphotoxin
Unfractionated PBLs	None	<8	<8
	LPS	20	<8
	BCG	82	<8
	BCG/LPS	86	<8
	BCG/LPS/PMA	140	<8
	PMA	280	<8
	SEB/Tα$_1$	100	10
	SEB/Tα$_1$/PMA	1,600	200
PBLs (adherent cells)	None	<8	<8
	BCG/LPS	350	<8
	SEB/Tα$_1$/PMA	590	<8
PBLs (non-adherent cells)	None	<8	<8
	BCG/LPS	<8	<8
	SEB/Tα$_1$/PMA	<8	350
HL-60	None	<8	<8
	PMA	380	<8
U-937	None	<8	<8
	PMA	32	<8

PBLs were obtained from plateletpheresis residues (Boston Red Cross) by Ficoll-Hypaque centrifugation[3]. Separation of PBLs into adherent and non-adherent populations was performed as described previously[41]. HL-60 (CCL 240) and U-937 cell lines (CRL 1593) were obtained from the American Type Culture Collection. Cells were suspended at 5×10^6 cells ml^{-1} in RPMI 1640 media containing 10% fetal bovine serum. Cultures were induced with one or more of the following agents: 2×10^5 organisms per ml of BCG (Calbiochem-Behring), 20 μg ml^{-1} *Salmonella typhimurium* lipopolysaccharide (LPS, Sigma), 1 μg ml^{-1} staphylococcal enterotoxin B (SEB, Sigma), 1 μg ml^{-1} thymosin α_1 (Tα_1)[42] and 10 ng ml^{-1} PMA (P-L Biochemicals). Cell-free supernatants were collected 24 h after induction except for the BCG/LPS and BCG/LPS/PMA treatments; for these two inductions a 24-h BCG stimulation was followed by an additional 24-h treatment with LPS and LPS/PMA, respectively. Samples were assayed for cytolytic activity on mouse L-929 fibroblasts as described previously[11]. The activities shown represent TNF-specific or lymphotoxin-specific units as determined after antibody neutralization at 4 °C for 4 h before assay. The units indicated were obtained from one representative donor in the case of the PBLs and from a single experiment when cell lines were used. Rabbit anti-human TNF antiserum was prepared against partially purified TNF from PBLs (L. Svedersky and T. Bringman, unpublished results). Rabbit anti-human lymphotoxin antiserum was prepared against purified human lymphotoxin from RPMI 1788 lymphoblastoid cells[11].

Here we identify a cell line with monocyte-like characteristics providing a source for human TNF and its messenger RNA. cDNA clones were isolated that encode a polypeptide related structurally to lymphotoxin. This cDNA was engineered to direct the synthesis of a relative molecular mass (M_r) 17,000 protein in *E. coli* with the immunological characteristics as well as *in vitro* and *in vivo* biological properties of natural human TNF.

A human TNF-producing cell line

We isolated PBLs by Ficoll-Hypaque density centrifugation and fractionated them into adherent monocytic and non-adherent lymphocytic fractions. After stimulation with BCG and endotoxin (lipopolysaccharide, LPS), we detected an activity cytotoxic to murine L-929 cells in the culture media of unfractionated mononuclear cells and monocytes (Table 1). No cytotoxic activity was produced by the non-adherent cells following the same BCG/LPS induction procedure. The failure of rabbit anti-human lymphotoxin antibodies to neutralize the cytotoxic activity demonstrates its difference from lymphotoxin. Moreover, the results of previous *in vivo* studies using BCG/LPS induction procedures[1,2] demonstrate that the activity can probably be attributed to TNF. Antiserum raised against partially purified PBL-produced TNF completely neutralized this activity (Table 1).

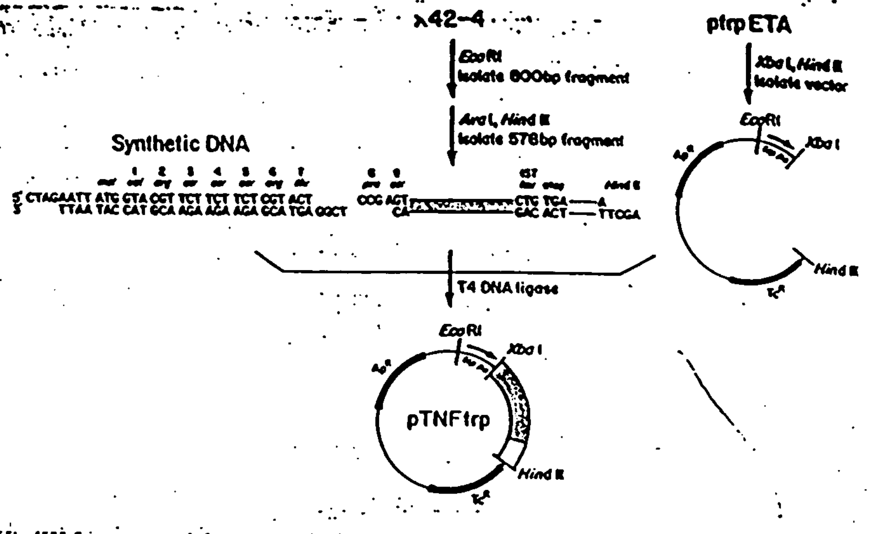

Fig. 2 Construction of a plasmid coding for the direct expression of mature human TNF in *E. coli*. The recombinant phage λ42-4 (10 μg) was digested with *Eco*RI and the 800-bp fragment containing the entire TNF coding region was isolated. TNF digestion with *Ava*I and *Hin*dIII gave a 578-bp fragment coding for amino acids 8–157. Two synthetic complementary deoxyoligonucleotides[17], 5′-dCTAGAATTATGGTACGTTCTTCTTCTCGTACT and 5′-dTCGGAGTACGAGAAGAAGAACGTACCATAAT, were designed to code for amino acids 1–7 of TNF, preceded by an ATG translational initiation codon, and to contain an *Xba*I cohesive terminus. The choice of codons for the first six amino acids of TNF was based on *E. coli* codon usage preferences[49]. An AATT sequence was incorporated upstream of the ATG to maximize expression by giving optimal spacing between the initiation codon and the *trp* leader Shine-Dalgarno sequence[50]. The pBR322-derived plasmid ptrpETA[51] was cleaved with *Hin*dIII and *Xba*I and the large fragment recovered by electroelution. The *Ava*I–*Hin*dIII fragment and the two synthetic deoxyoligonucleotides were inserted into the plasmid pTNFtrp expression vector to give the plasmid pTNFtrp. The methods used to assemble the fragments and verify the construction of pTNFtrp have been described previously[19,20]. *E. coli* W3110/pTNFtrp was grown in M9 medium containing 5 μg ml^{-1} tetracycline to 0.2 A_{550} units. Indole acrylic acid was added to a concentration of 20 μg ml^{-1}. The cells were collected at A_{550} = 1.0 and washed with cold PBS. The final cell pellet was resuspended in 1 ml PBS, sonicated on ice for 30 s and the resulting extract diluted in PBS for assay on L-929 cells[11].

Yields of adherent cells from peripheral blood were low and s levels f TNF produced were variable and donor-dependent; therefore tested alternative induction schemes for the produc- n of TNF from total PBLs (Table 1). An increase in cytotoxic tivity was observed when the PBLs were co-stimulated with *aphylococcus* enterotoxin B, desacetyl-thymosin-α_1 and the mour-promoting agent 4β-phorbol 12β-myristate 13α-acetate MA). However, antibody neutralization experiments demon- ated that a significant portion of measured activity was lym- otoxin. Therefore, we screened a number of transformed cell es of haematopoietic origin for their ability to synthesize NF. Activity which could be neutralized by anti-TNF anti- rum was detected following PMA treatment in two monocyte- e cell lines, HL-60, derived from a promyelocytic leukaemia[14], d U-937, derived from a histiocytic lymphoma[15] (Table 1). e HL-60 cell line consistently produced higher TNF titres 00–400 U ml^{-1} 24 h after induction) than the U-937 cell line :100 U ml^{-1}). A time course of TNF synthesis by HL-60 cul- res indicated that measurable activity was detected 2 h after A treatment (data not shown). Therefore the HL-60 cell line s selected for future experiments; supernatants were collected –24 h after induction for protein purification and 4-h induc- ns were used when cells were collected for RNA isolation.

NF cDNA clone identification

man TNF was purified to homogeneity from filtrates of A-stimulated HL-60 cell cultures (see ref. 16). A single mponent f M_r 17,000 was observed when the purified TNF s analysed by SDS-gel electrophoresis in reducing conditions. obtain amin acid sequence information, tryptic peptides of NF were prepared and separated by reverse-phase HPLC. The preliminary sequence Glu-Thr-Pro-Glu-Gly-Ala-Glu- a-Lys-Pro-Trp-Tyr-Glu-Lys was determined for the first tryp- fragment (TD-6) analysed. A single synthetic 42-base long oxyolig nucleotide (42-mer) which could code for this amino id sequence was chemically synthesized[17] for use as a hybridiz- ion probe. The design f the probe sequence (dGAAACCCCT- AAGGGGCTGAAGCCAAGCCCTGGTATGAAAAG) was sed on published human codon usage frequencies[18] and the don bias of human γ-interferon[19], tissue-type plasminogen tivator[20] and lymphotoxin[12]. The general usefulness of this ng probe' approach has been demonstrated recently by the entification of several cloned genomic DNA sequences[21–23] d cDNAs[24,25].

An oligo(dT)-primed HL-60 cDNA library of ~200,000 clones prepared in λgt10 (ref. 26) was screened with the ^{32}P-labelled 42-mer. The nine distinct phage which gave positive signals with this probe were hybridized with 'induced' and 'non-induced' ^{32}P-labelled cDNA probes[19] prepared using poly(A) mRNA obtained from 4-h PMA-treated and untreated HL-60 cultures, respectively. Seven of these recombinant phage DNAs hybridized weakly to the induced probe but did not hybridize to the uninduced probe, as expected for authentic TNF cDNAs. Restriction endonuclease mapping indicated that these seven cDNA clones were related to each other and that the phage λ42-4 contained the longest cDNA insert.

TNF cDNA sequence

We determined the sequence of the 1,300 base pair (bp) cDNA insert of phage λ42-4 (nucleotides 337–1,643; Fig. 1). Alignment of the cDNA sequence with the 42-mer probe sequence gave the proper reading frame of the cDNA and demonstrated that it did indeed encode TNF. Of the 14 amino acids (residues 104–117, Fig. 1) assigned to tryptic peptide TD-6 on the basis of preliminary protein sequence, 13 were correct; the only discrepancy was in the last amino acid (position 117) where the cDNA sequence encodes a proline residue rather than the predicted lysine. Despite this difference, the hybridization of the synthetic probe to the TNF cDNA clone was successful, as the 42-mer matched the cDNA sequence in 34 of the first 38 positions, including a stretch of 17 consecutive homologous nucleotides (nucleotides 711–727; Fig. 1).

The assignment of valine (residue 1, Fig. 1) as the first residue of mature TNF was based on NH_2-terminal protein sequence analysis of the intact molecule (Val-Arg-Ser-Ser-Ser---)[16]. There are 156 amino acids encoded after this valine before an in-phase termination codon occurs. The coding region of TNF is followed by 792 nucleotides of 3′ untranslated sequence containing the hexanucleotide AATAAA (position 1,630–1,635) which precedes the site of polyadenylation in most eukaryotic mRNAs[27].

Additi nal confirmation that this sequence codes for TNF was obtained by determining the amin acid sequence of nine tryptic peptides of natural HL-60 TNF and several peptides generated by digesti n with *S. aureus* V8 protease and chymotrypsin[16]. The M_r of 17,356 calculated f r the mature TNF mon mer from the cDNA sequence agrees closely with the value obtained for natural TNF by SDS-polyacrylamide gel electrophoresis and amino acid compositi n[16]. These results and

Table 2 Necrosis of MethA sarcoma in vivo by TNF

Treatment	+++	++	+	−
	Necrotic response (No. of mice)			
PBS, i.l.	0	1	1	9
PBS, i.p.	0	0	1	4
PBS, i.m.	0	0	1	4
E. coli LPS, i.l.	0	0	1	9
HL-60 TNF, i.l.	5	1	1	0
rTNF, i.l.	7	5	0	0
rTNF, i.p.	2	1	2	0
rTNF, i.m.	2	2	1	0

(BALB/c×C57BL/6)F_1 female mice were injected intradermally with 5×10^5 BALB/c MethA sarcoma cells. Ten days later, the tumours (0.75 cm average diameter) were injected intralesionally (i.l., 1×10^5 U), intraperitoneally (i.p., 5×10^5 U) or intramuscularly (i.m., 5×10^5 U) with TNF in a total volume of 0.1 ml PBS. At 24 h after TNF treatment the tumours were excised, sectioned and scored for haemorrhagic necrosis by visual and histological examination as described previously[1]. In the maximum response (+++) 50–75% of the tumour mass is markedly necrotic after 24 h; ++ denotes a moderate response, that is 25–50% haemorrhagic necrosis; +, a minimal response of <25% haemorrhagic necrosis; −, tumours showed no visible necrosis. Natural TNF was purified from HL-60 cultures as described elsewhere[16]. Recombinant TNF (rTNF) was purified from *E. coli* W3110/pTNFtrp to a purity of >95% and a specific activity of $\sim10^8$ units mg^{-1} (T. Bringman, unpublished results).

the absence of any potential *N*-glycosylation sites in the deduced amino acid sequence suggest that TNF is not a glycoprotein. These data suggest also that TNF may occur naturally in multimeric form, as the M_r estimated previously for human TNF ranged from 34,000–140,000 (refs 6, 28). There are two cysteine residues (positions 69 and 101) in TNF which are probably involved in a single intramolecular disulphide bond[16].

The cDNA clone λ42-4 contains the entire coding region of mature TNF but lacks a complete signal peptide coding sequence and initiation codon. To obtain the missing sequence information, a specifically-primed cDNA library was prepared (see Fig. 1 legend) and screened with the ^{32}P-labelled λ42-4 cDNA insert. A cDNA clone (λ16-4) was identified which contained an insert extending 337 bp further 5′ than the λ42-4 insert (Fig. 1).

From an analysis of the TNF cDNA sequence, it seems that TNF is synthesized initially as part of a larger precursor (pre-TNF). Starting at the 5′ end of the cDNA, 125 nucleotides of non-translated sequence are followed by a methionine codon and an open reading frame of 233 amino acids. This AUG is preceded by termination codons in all three frames, suggesting that it is the initiation codon. Furthermore, the sequence context of this AUG conforms closely to the CC^A_GCC<u>AUG</u>G proposed[29] as a consensus sequence for eukaryotic initiator sites.

The presequence of 76 residues is most probably involved in the secretion of TNF as it is not observed on the mature TNF polypeptide and contains an unusually long hydrophobic region of 26 amino acids (residues −46 to −21). Typically, signal peptides involved in protein secretion are only 20–30 amino acids long[30,31]. However, a signal sequence for the Rous sarcoma virus envel pe glycoprotein[32] is atypically long (63 residues) and contains also many charged amino acids at its amino terminus, such as pre-TNF. It is interesting to note the presence of Arg-Arg and Lys-Lys dipeptides in the first 30 amino acids of the TNF pre-sequence, as pairs of basic amino acids often serve as cleavage sites for the release of physiologically-important peptides from precursor molecules[33-36].

We used the ^{32}P-labelled λ42-4 cDNA insert to examine TNF gene structure and mRNA size. Results from Southern[37] hybridizations indicate that only a single gene f r TNF is present in the human gen me. Northern hybridizati n analysis[38] shows that a single mRNA species ~18S in size is synthesized in PMA-induced HL-60 cultures and BCG/LPS-treated macrophages isolated from human PBLs. This provides additional evidence that the same cytot xin is produced from both

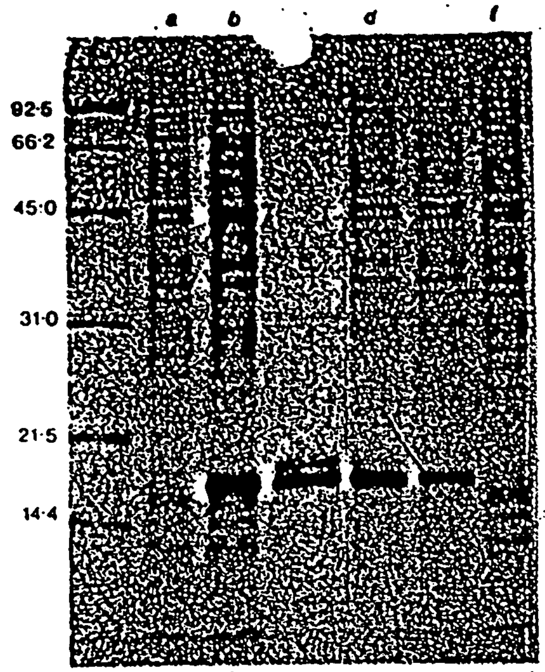

Fig. 3 SDS-polyacrylamide gel electrophoresis of human TNF synthesized in *E. coli*. *E. coli* K-12 strain W3110, transformed with pTNFtrp or pBR322, was grown in M9 medium containing 5 μg ml^{-1} tetracycline. Cells were collected, lysed in 2% SDS, 1% β-mercaptoethanol and precipitated with 10 volumes of cold acetone. Samples were electrophoresed on a 12.5% SDS-polyacrylamide slab gel using the buffer system of Maizels[52] and the gel stained with Coomassie brilliant blue. The left lane contains protein M_r standards ($\times10^{-3}$): phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,500). Lanes *a*, *f*, cell lysates of *E. coli* W3110/pBR322; lanes *b*, *e*, cell lysates of *E. coli* W3110/pTNFtrp; lane *c*, partially purified human TNF isolated from the HL60 cell line[16]; lane *d*, mixture of the *E. coli* W3110/pTNFtrp cell lysate and the HL60-derived, purified TNF.

cell sources and suggests that the TNF cDNA sequence shown in Fig. 1 represents a nearly full-length copy of TNF mRNA. No hybridization was detected to mRNA isolated from uninduced cultures (data not shown).

TNF synthesis in *E. coli*

Proof that the cDNA described here encodes TNF requires the demonstration that it can direct the synthesis of a gene product with the properties of authentic human TNF. To allow characterization of the protein encoded by the cloned cDNA, we engineered the TNF cDNA sequence for direct expression in *E. coli* (Fig. 2). In the resulting expression plasmid, pTNFtrp, the TNF DNA sequence is under the transcriptional control of a 300-bp DNA fragment of the *E. coli trp* operon containing the *trp* promoter, operator and Shine–Dalgarno sequence of the *trp* leader peptide.

Total extracts of *E. coli* K-12 strain W3110 transformed with pTNFtrp contained a prominent polypeptide with an apparent M_r 17,000 (Fig. 3, lanes *b*, *e*). This protein is not visible in cells transformed with pBR322 (lanes *a*, *f*), strong evidence that it represents the translational product of the TNF cDNA sequence. Furthermore, this protein co-migrates with authentic TNF (lane *c*) isolated from the HL-60 cell line (lane *d*), suggesting that no significant post-translational processing of TNF occurs in the HL-60 cell line. This is unlike lymph toxin and γ-interferon, both of which occur naturally as heterogene us glycoproteins as a consequence of N-terminal[12] and C-terminal[39] proteolysis, respectively.

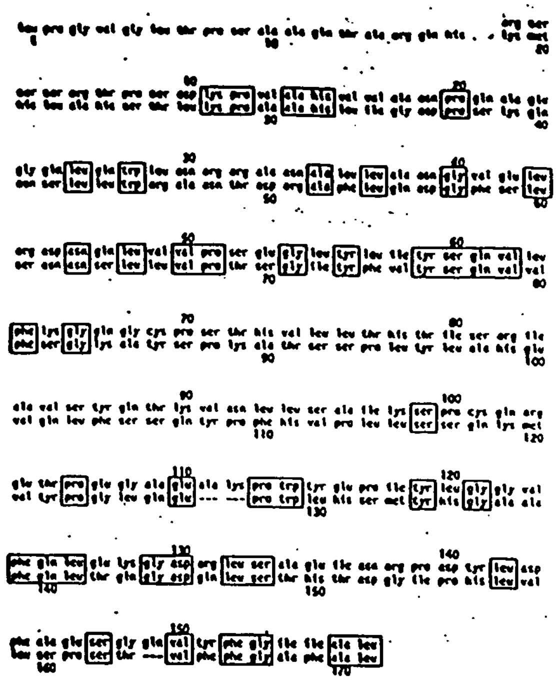

Fig. 4 Comparison of the amino acid sequence of human TNF with human lymphotoxin[12,13]. The sequences have been aligned to give maximal homology by introducing two gaps (indicated by dashed lines) into the lymphotoxin sequence. Identical amino acids are boxed. The numbers above each row (1-157) and below each row (1-171) indicate the amino acids of mature TNF and lymphotoxin (LT), respectively.

Verification of the bacterial production of biologically-active TNF was obtained by assaying extracts of *E. coli* W3110/pTNFtrp for cytolytic activity in the murine L-929 fibroblast assay[1]. Approximately 300,000 units of activity were detected per ml of culture at $A_{550} = 1$, whereas no activity was observed in *E. coli* W3110/pBR322 controls. This corresponds to ~3 mg of active TNF per l ($A_{550} = 1$) or about 300,000 molecules of active TNF per cell if a specific activity of 10^8 units mg^{-1} (ref. 16) is assumed. The activity was neutralized by antiserum prepared against partially purified PBL-derived TNF, but was not neutralized by preimmune serum or rabbit anti-human lymphotoxin antibodies (data not shown).

In vivo necrosis activity

TNF is generally defined as a cytotoxin released by BCG/LPS-treated macrophages which induces the haemorrhagic necrosis of methylcholanthrene-induced (MethA) sarcomas in BALB/c mice[1,2]. Therefore, we examined recombinant human TNF purified from *E. coli* and natural human TNF from PMA-induced HL-60 cultures for *in vivo* tumour necrosis activity in the MethA assay[1]. Both recombinant and natural TNF samples elicited significant necrotic responses, regardless of whether the TNF was injected intralesionally or systemically (Table 2). Minimal or no necrosis of the MethA sarcoma tumours was observed in mice injected with either phosphate-buffered saline (PBS) or 100 µg *E. coli* LPS. These results, taken with the antibody neutralization and Northern hybridization data, provide further evidence that the cytotoxin described here is human TNF.

Homology to lymphotoxin

The known *in vivo* and *in vitro* biological activities of TNF and lymphotoxin are very similar[2,3,13]. TNF and lymphotoxin are now known to be antigenically distinct molecules[3]. It has thus become common to distinguish these two lymphokines on the basis of the cell populations responsible for their synthesis. We have compared the amino acid sequences of human TNF and lymphotoxin to determine whether similarities in their biological properties might be attributed to common structural features (Fig. 4). By introducing two gaps, the lymphotoxin sequence can be aligned with the TNF sequence so that distinct homologies are apparent; we find 44 of the 157 TNF residues (28%) are identical to corresponding lymphotoxin amino acids with many of the remaining differences between the two polypeptides resulting from conservative amino acid changes. The nucleotide homology over this coding region is 46% (data not shown). Two particularly conserved regions occur at amino acids 35-66 and 110-133 (TNF numbering) where 50% of the residues (28 of 56) are identical for TNF and lymphotoxin. The hydrophobic carboxy-termini of the two molecules are also significantly conserved. It is probable that the conserved regions are crucial to the shared cytotoxic activities of TNF and lymphotoxin, perhaps through interaction with a common receptor expressed on the surface of transformed cells. Support for this hypothesis is provided by the lack of cytotoxic activity in a truncated lymphotoxin polypeptide lacking its last 16 amino acids[13].

Lymphotoxin has 18 more NH_2-terminal amino acids than TNF (Fig. 4), suggesting that this region is not required for cytotoxic activity. In fact, a 148 residue lymphotoxin, consisting of amino acids 24-171 of mature lymphotoxin, and having similar cytotoxic effects on L-929 cells, has been isolated from the RPMI-1788 cell line[11,12]. It is also interesting that amino acids 67-109 of TNF are unrelated to the corresponding region of lymphotoxin; only two of 43 residues are identical. This region includes all of the amino acids spanned by the Cys 69-Cys 101 disulphide bridge of TNF. One possible role for this non-conserved region could be to position correctly the two surrounding homologous regions in a conformation essential for cytotoxic activity. Such positioning, which could be achieved by a TNF disulphide bond, may require a very different sequence of amino acids in lymphotoxin, where no disulphide bond exists. These apparently unrelated regions of TNF and lymphotoxin might specify also as yet undiscovered differences in biological activities and/or target sites between the two molecules. The availability of efficient expression systems for TNF and lymphotoxin[13], in combination with the techniques of site-directed mutagenesis[40], will make it possible to address questions of this type directly.

We thank Phil Hass for growing HL-60 cells; Dr Lloyd Svedersky and Tim Bringman for preparing TNF antiserum; Mark Vasser, Parkash Jhurani and Peter Ng for deoxyoligonucleotide synthesis; Irene Figari and Refaat Shalaby for assistance with the tumour necrosis assays; Roxanne Chang and the Genentech Bioassay Group for performing *in vitro* TNF assays; and Dr Richard Harkins for helpful suggestions. G.E.N. dedicates this work to the late Jack L. Levenson.

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Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing

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A novel mammalian neuropeptide, the tachykinin substance K, is specified by a discrete genomic segment. Alternative RNA splicing generates two distinct mRNAs encoding the neuropeptide substance P alone or with substance K from a single preprotachykinin gene. Relative amounts of the mRNAs vary in different tissues, suggesting that the substance K-encoding sequence is regulated in a tissue-specific manner.

SUBSTANCE P is one of the best characterized neuropeptides in mammalian tissues; several lines of evidence suggest that it acts as a neurotransmitter or neuromodulator in primary sensory neurones[1]. Substance P belongs to a family of related peptides, the tachykinins, and is thought to be the only member of this family present in mammalian tissues[2]. Recently, we elucidated the entire primary structures of two types of bovine brain substance P precursors (α- and β-preprotachykinins) by determining their cloned cDNA sequences[3]. β-Preprotachykinin (β-PPT) contains not only the substance P sequence but also a novel tachykinin sequence designated substance K, whereas α-preprotachykinin (α-PPT) lacks the latter sequence, containing only substance P. The decapeptide substance K has been found independently as neurokinin α, a gut-contracting peptide in porcine spinal cord[4]. The chemically synthesized substance K peptide possesses biological activities characteristic of the tachykinin family, but is considerably more potent than substance P in some pharmacological tests[5,6]. Substance K thus represents a second type of mammalian tachykinin which may have a physiological role different from that of substance P in mammalian organisms.

The two PPT mRNAs exhibit an interesting structural relationship. They have complete identity in their 5′ and 3′ sequences and differ only in the insertion/deletion of the sequence coding for the substance K region[3]. This characteristic structural relationship poses intriguing questions about the gene organization for these two mRNAs and the regulation for the generation of the two biologically different mammalian tachykinins. Our present investigations thus concern the structural organization of the PPT gene and the distribution and regulation of the two PPT mRNAs in the nervous system and peripheral tissues. We report here that the sequence specifying the substance K region is encoded by a discrete genomic segment, and that both α- and β-PPT mRNAs arise from a single gene by alternative RNA splicing events. We also present evidence indicating the tissue-specific regulation of the PPT gene for the differential generation of the two PPT mRNAs.

PPT gene organization

Genomic clones containing the bovine preprotachykinin gene were isolated from a bovine genomic library by hybridization *in situ* with a bovine β-PPT cDNA probe, and all the isolated genomic DNA fragments were arranged into an approximately 36 kilobase-pair (kbp) length of a continuous genomic DNA (Fig. 1*a*; see Fig. 1 legend for experimental details of cloning). Nucleotide sequence analysis was performed on DNA fragments containing exons and their surrounding regions (Fig. 1 *b–f*). Comparison of the genomic DNA sequence with the cDNA sequence enabled us to construct a structural organization of the bovine PPT gene (Fig. 1*g*). Intron A (403 base pairs, bp) is inserted within the segment encoding the 5′-untranslated region of the mRNA, 9–10 bp upstream from the translational initiation site. Introns B (~1.0 kbp), C (~450 bp), D (~460 bp), E (~1.4 kbp) and F (~3.6 kbp) all interrupt the protein-encoding region of the gene. The sequences at the exon–intron boundaries are consistent with the splice junction sequences observed for other genes[7]. Exons 2–7 consist of 132, 97, 45, 24, 54 and 596 bp, each encoding the protein sequence corresponding to the signal peptide, substance P, two spacer sequences, substance K, and the C-terminal sequence, respectively. It is remarkable that exon 6 precisely specifies the substance K region missing in α-PPT. Because blot-hybridization analysis of total cellular DNAs (data not shown) as well as the genomic cloning described above showed that no more than one PPT gene is present in the bovine genome, we conclude that both α- and β-PPT mRNAs are produced from a single gene as a consequence of alternative RNA splicing events.

The 5′ termini of the PPT mRNAs were identified by S_1 nuclease mapping and primer extension analyses (Fig. 2). Both analyses revealed a length heterogeneity at the 5′ end of the PPT transcripts. The major 5′ termini of the PPT mRNAs mapped at 106–108, 110 and 111 bp upstream from the 3′ end of exon 1 (Fig. 1*g*). Several minor mRNA species starting further upstream were also observed and these 5′ termini mapped at roughly 132, 133, 137 and 146 bp upstream from the 3′ end of exon 1. In support of these assignments, we found that three of the four cDNA clones isolated previously[3] (clones pSP301, 302 and 306) contained the extreme 5′ sequences corresponding to the major 5′ ends, while the remaining one (clone pSP307) extended its 5′-terminus up to one of the minor 5′ ends. Based on the assignments of the 5′ termini of the PPT mRNAs, we conclude that the bovine PPT gene is ~8.4 kbp long.

Entrez Protein

PubMed Nucleotide Protein Genome Structure PopSet Taxonomy OMIM

Search Protein for Go Clear

Limits Index History Clipboard

Display Default View as HTML Save Add to Clipboard

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BLink, PubMed, Related Sequences, Taxonomy, OMIM

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  AUTHORS   Pennica,D., Nedwin,G.E., Hayflick,J.S., Seeburg,P.H., Derynck,R.,
            Palladino,M.A., Kohr,W.J., Aggarwal,B.B. and Goeddel,D.V.
  TITLE     Human tumour necrosis factor: precursor structure, expression and
            homology to lymphotoxin.
  JOURNAL   Nature 312 (5996), 724-729 (1984)
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  AUTHORS   Aggarwal,B.B., Kohr,W.J., Hass,P.E., Moffat,B., Spencer,S.A.,
            Henzel,W.J., Bringman,T.S., Nedwin,G.E., Goeddel,D.V. and
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  TITLE     Molecular cloning of the complementary DNA for human tumor necrosis
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  JOURNAL   Science 228 (4696), 149-154 (1985)
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  TITLE     Human lymphotoxin and tumor necrosis factor genes: structure,
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  JOURNAL   Nucleic Acids Res. 13 (17), 6361-6373 (1985)
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            et,al.
  TITLE     Molecular cloning and expression of human tumor necrosis factor and
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  JOURNAL   Eur. J. Biochem. 152 (3), 515-522 (1985)
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            Nakamura, K.I., Ando, O., Torigoe, K., Sugimoto, T. and Kurimoto,
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  TITLE     Simultaneous production of natural human tumor necrosis
            factor-alpha, -beta and interferon-alpha from BALL-1 cells
            stimulated by HVJ
  JOURNAL   Lymphokine Res. 7 (2), 175-185 (1988)
  MEDLINE   88301617
REFERENCE   7  (residues 1 to 233)
  AUTHORS   Stevenson,F.T., Bursten,S.L., Locksley,R.M. and Lovett,D.H.
  TITLE     Myristyl acylation of the tumor necrosis factor alpha precursor on
            specific lysine residues
  JOURNAL   J. Exp. Med. 176 (4), 1053-1062 (1992)
  MEDLINE   93018820
  REMARK    annotation; identification of myristylated lysines
REFERENCE   8  (residues 1 to 233)
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  TITLE     Dense Alu clustering and a potential new member of the NFkappaB
            family within a 90 kilobase HLA class III segment
  JOURNAL   Nature Genet. 3, 137-145 (1993)
REFERENCE   9  (residues 1 to 233)
  AUTHORS   D'Alfonso,S. and Richiardi,P.M.
  TITLE     A polymorphic variation in a putative regulation box of the TNFA
            promoter region
  JOURNAL   Immunogenetics 39 (2), 150-154 (1994)
  MEDLINE   94102809
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  AUTHORS     Nedwin,G.E., Naylor,S.L., Sakaguchi,A.Y., Smith,D.,
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  TITLE       Human lymphotoxin and tumor necrosis factor genes: structure,
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              Arsdell,J.N., Yamamoto,R. and Mark,D.F.
  TITLE       Molecular cloning of the complementary DNA for human tumor necrosis
              factor
  JOURNAL     Science 228 (4696), 149-154 (1985)
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  AUTHORS     Marmenout,A., Fransen,L., Tavernier,J., van der Heyden,J.,
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  TITLE       Molecular cloning and expression of human tumor necrosis factor and
              comparison with mouse tumor necrosis factor
  JOURNAL     Eur. J. Biochem. 152 (3), 515-522 (1985)
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REFERENCE     7  (residues 1 to 233)
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              and Cohen,D.
  TITLE       Dense Alu clustering and a potential new member of the NF kappa B
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  JOURNAL     Nat. Genet. 3 (2), 137-145 (1993)
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  TITLE       Direct Submission
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  TITLE       Structure of tumour necrosis factor
  JOURNAL     Nature 338 (6212), 225-228 (1989)
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  TITLE       The structure of tumour necrosis factor--implications for
              biological function
  JOURNAL     J. Cell Sci. Suppl. 13, 11-18 (1990)
  MEDLINE     91193276
  REMARK      X-RAY CRYSTALLOGRAPHY (2.9 ANGSTROMS).
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  AUTHORS     Eck,M.J. and Sprang,S.R.
  TITLE       The structure of tumor necrosis factor-alpha at 2.6 A resolution.
              Implications for receptor binding
  JOURNAL     J. Biol. Chem. 264 (29), 17595-17605 (1989)
  MEDLINE     90008932
  REMARK      X-RAY CRYSTALLOGRAPHY (2.6 ANGSTROMS).
REFERENCE     12 (residues 1 to 233)
  AUTHORS     Reed,C., Fu,Z.Q., Wu,J., Xue,Y.N., Harrison,R.W., Chen,M.J. and
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            Weber,I.T.
  TITLE     Crystal structure of TNF-alpha mutant R31D with greater affinity
            for receptor R1 compared with R2
  JOURNAL   Protein Eng. 10 (10), 1101-1107 (1997)
  MEDLINE   98147459
  REMARK    X-RAY CRYSTALLOGRAPHY (2.3 ANGSTROMS) OF MUTANT ARG-107.
REFERENCE   13 (residues 1 to 233)
  AUTHORS   Cha,S.S., Kim,J.S., Cho,H.S., Shin,N.K., Jeong,W., Shin,H.C.,
            Kim,Y.J., Hahn,J.H. and Oh,B.H.
  TITLE     High resolution crystal structure of a human tumor necrosis
            factor-alpha mutant with low systemic toxicity
  JOURNAL   J. Biol. Chem. 273 (4), 2153-2160 (1998)
  MEDLINE   98113178
  REMARK    X-RAY CRYSTALLOGRAPHY (1.8 ANGSTROMS) OF MUTANT M3S.
REFERENCE   14 (residues 1 to 233)
  AUTHORS   Van Ostade,X., Tavernier,J., Prange,T. and Fiers,W.
  TITLE     Localization of the active site of human tumour necrosis factor
            (hTNF) by mutational analysis
  JOURNAL   EMBO J. 10 (4), 827-836 (1991)
  MEDLINE   91184128
  REMARK    MUTAGENESIS.
REFERENCE   15 (residues 1 to 233)
  AUTHORS   Stevenson,F.T., Bursten,S.L., Locksley,R.M. and Lovett,D.H.
  TITLE     Myristyl acylation of the tumor necrosis factor alpha precursor on
            specific lysine residues
  JOURNAL   J. Exp. Med. 176 (4), 1053-1062 (1992)
  MEDLINE   93018820
  REMARK    MYRISTOYLATION.
COMMENT     -------------------------------------------------------------------
            This SWISS-PROT entry is copyright. It is produced through a
            collaboration between the Swiss Institute of Bioinformatics and
            the EMBL outstation - the European Bioinformatics Institute.
            The original entry is available from http://www.expasy.ch/sprot
            and http://www.ebi.ac.uk/sprot
            -------------------------------------------------------------------
            [FUNCTION] TNF IS MAINLY SECRETED BY MACROPHAGES, IT IS A CYTOKINE
            WITH A WIDE VARIETY OF FUNCTIONS: IT CAN CAUSE CYTOLYSIS OF CERTAIN
            TUMOR CELL LINES, IT IS IMPLICATED IN THE INDUCTION OF CACHEXIA, IT
            IS A POTENT PYROGEN CAUSING FEVER BY DIRECT ACTION OR BY
            STIMULATION OF INTERLEUKIN 1 SECRETION, IT CAN STIMULATE CELL
            PROLIFERATION AND INDUCE CELL DIFFERENTIATION UNDER CERTAIN
            CONDITIONS.
            [SUBUNIT] HOMOTRIMER.
            [SUBCELLULAR LOCATION] TYPE II MEMBRANE PROTEIN. ALSO EXISTS AS AN
            EXTRACELLULAR SOLUBLE FORM.
            [PTM] THE SOLUBLE FORM DERIVES FROM THE MEMBRANE FORM BY
            PROTEOLYTIC PROCESSING.
            [DISEASE] CACHEXIA ACCOMPANIES A VARIETY OF DISEASES, INCLUDING
            CANCER AND INFECTION, AND IS CHARACTERIZED BY GENERAL ILL HEALTH
            AND MALNUTRITION.
            [SIMILARITY] BELONGS TO THE TUMOR NECROSIS FACTOR FAMILY.
FEATURES             Location/Qualifiers
     source          1..233
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
     Region          1..76
                     /region_name="Propeptide"
     Protein         1..233
                     /product="TUMOR NECROSIS FACTOR PRECURSOR"
                     1..233
     Site            19
                     /site_type="lipid-binding"
                     /note="MYRISTATE."
     Site            20
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                     /site_type="lipid-binding"
                     /note="MYRISTATE."
     Region          36..56
                     /region_name="Transmembrane region"
                     /note="SIGNAL-ANCHOR (TYPE-II MEMBRANE PROTEIN)."
     Region          63
                     /region_name="Conflict"
                     /note="F -> S (IN REF. 5)."
     Region          77..233
                     /region_name="Mature chain"
                     /note="TUMOR NECROSIS FACTOR."
     Region          85..86
                     /region_name="Hydrogen bonded turn"
     Region          89..94
                     /region_name="Beta-strand region"
     Region          99..100
                     /region_name="Hydrogen bonded turn"
     Region          104..105
                     /region_name="Beta-strand region"
     Site            105
                     /site_type="mutagenized"
                     /note="L->S: LOW ACTIVITY."
     Site            108
                     /site_type="mutagenized"
                     /note="R->W: BIOLOGICALLY INACTIVE."
     Region          109..110
                     /region_name="Hydrogen bonded turn"
     Region          112..113
                     /region_name="Beta-strand region"
     Site            112
                     /site_type="mutagenized"
                     /note="L->F: BIOLOGICALLY INACTIVE."
     Region          115..116
                     /region_name="Hydrogen bonded turn"
     Region          118..119
                     /region_name="Beta-strand region"
     Region          124..125
                     /region_name="Beta-strand region"
     Region          132..138
                     /region_name="Beta-strand region"
     Region          140..142
                     /region_name="Beta-strand region"
     Bond            bond(145,177)
                     /bond_type="disulfide"
     Region          152..159
                     /region_name="Beta-strand region"
     Site            160
                     /site_type="mutagenized"
                     /note="A->V: BIOLOGICALLY INACTIVE."
     Site            162
                     /site_type="mutagenized"
                     /note="S->F: BIOLOGICALLY INACTIVE."
     Region          166..174
                     /region_name="Beta-strand region"
     Site            167
                     /site_type="mutagenized"
                     /note="V->A,D: BIOLOGICALLY INACTIVE."
     Region          190..191
                     /region_name="Beta-strand region"
     Region          194..200
                     /region_name="Beta-strand region"
     Region          207..212
                     /region_name="Beta-strand region"
     Region          215..217
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                     /region_name="Helical region"
     Region          218
                     /region_name="Beta-strand region"
     Site            222
                     /site_type="mutagenized"
                     /note="E->K: BIOLOGICALLY INACTIVE."
     Region          224..225
                     /region_name="Hydrogen bonded turn"
     Region          226..232
                     /region_name="Beta-strand region"
ORIGIN
        1 mstesmirdv elaeealpkk tggpqgsrrc lflslfsfli vagattlfcl lhfgvigpqr
       61 eefprdlsli splaqavrss srtpsdkpva hvvanpqaeg qlqwlnrran allangvelr
      121 dnqlvvpseg lyliysqvlf kgqgcpsthv llthtisria vsyqtkvnll saikspcqre
      181 tpegaeakpw yepiylggvf qlekgdrlsa einrpdyldf aesgqvyfgi ial
//
```

Peptide Synthesis Calculation Sheet:

Notebook name is "s309".
Notebook file is "S309.NBK".

Target Peptide: length = 22, MW = 2507.037

NH2-His-Val-Leu-Leu-Thr-His-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Tyr-
Gln-Thr-Lys-Val-Asn-Leu-Leu-COOH

Resin substitution = 0.100 meq/g
Resin quantity = 3.000 g
Excess amino acid = 4.000 x

Sequence checked
23.10.89
J.Y

Peptide Quantity = 0.300 mMoles
Theoretical Yield = 0.752 g

Starting Resin: FMOC-Leu-PepSyn-KA

Difficult to remove Arg... to the peptide

Peptide Recycling S343

Peptide Synthesis Calculation Sheet:

Notebook name is "s323".
Notebook file is "S323.NBK".

Target Peptide: length = 11, MW = 1238.416

NH2-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Tyr-Gln-Thr-COOH

Resin substitution	=	0.100	meq/g
Resin quantity	=	4.000	g
Excess amino acid	=	4.000	x
Peptide Quantity	=	0.400	mMoles
Theoretical Yield	=	0.495	g

Sequence checked
21.11.89

Starting Resin: FMOC-Thr-PepSyn-KA

PEPTIDE TECHNOLOGY LTD
SYNTHESIS NO. 323 14 mg
H-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Tyr-Gln-Thr-OH
STORE BELOW 5 DEG....NOT FOR HUMAN USE

Exhibit 2